Cross-species transferability of microsatellite markers in the genus *Lippia*

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**ABSTRACT.** The cross-species transferability of 20 microsatellite markers was tested in the genus *Lippia*. Eleven markers were polymorphic after screening 19 accessions of *Lippia sidoides* and *Lippia gracilis* maintained in the Active Germplasm Bank (AGB) from Universidade Federal de Sergipe. Additionally, 40 accessions of *Lippia* spp were collected in Sergipe to increase the germplasm bank. A total of 23, 22, and 36 alleles were identified, with an average of 2.3, 2.2, and 3.27 alleles per locus, respectively, for each group. The markers that were used were efficient tools to
access genetic diversity in the germplasm bank and will be useful for further research aiming at the conservation and management of these important aromatic species.

**Key words:** Genetic diversity; *Lippia sidoides*; *Lippia gracilis*; *Lippia alba*; Microsatellite markers; Transferability

**INTRODUCTION**

The genus *Lippia* consist of about 200 species of herbs, shrubs, and small trees of vegetative propagation, which are often aromatic (Terblanché and Kornelius, 1996). In this genus, the aromatic species *Lippia sidoides* and *Lippia gracilis*, which are native of northeastern Brazilian, might be highlighted because of the strong bactericidal, fungicidal, and acaricidal activity that is conferred by their essential oil because of the predominance of the monoterpenes thymol and carvacrol (Albuquerque et al., 2006; Botelho et al., 2007; Cruz et al., 2013).

The Universidade Federal de Sergipe (UFS) has a germplasm collection of *L. sidoides* and *L. gracilis*. However, there is no information about its genetic diversity for the management of these germplasms and future breeding programs. In this sense, microsatellite markers, or simple sequence repeats (SSRs), have been an efficient alternative because of their codominance, polymorphic and multi-allelic nature, abundance, and wide coverage of the genome (Buso et al., 2003). Because the development of SSR primers for each locus is associated with high costs and labor intensity, these markers are mainly available for some economically important crops. However, closely related species have conserved sequences that flank SSRs, which makes it possible to share primers among species (Kuleung et al., 2004). In this study, we determined the transferability of a set of markers that were developed and characterized for *Lippia alba* (Mill) N. E. Br. to characterize the genetic diversity of the Active Germplasm Bank (AGB) of *L. sidoides* and *L. gracilis*.

**MATERIAL AND METHODS**

Young leaves of 12 accessions of *L. sidoides* and 7 accessions of *L. gracilis* that were maintained in the AGB of UFS were collected for DNA extraction. Because of the small number of genotypes, a set of 40 accessions of *Lippia* spp was collected for the introduction of new divergent genotypes to expand the AGB. The plant material was immediately stored in liquid nitrogen and lyophilized. After lyophilization, the plant material was ground and stored at -20°C. DNA extraction was performed according to the cetyltrimethylammonium bromide protocol described by Doyle and Doyle (1990). The quantity and quality of the obtained DNA were analyzed on 1% agarose gels that were stained with SYBR safe using different concentrations of phage lambda DNA.

A set of 11 markers that were characterized by Santos et al. (2012) and 9 microsatellite markers characterized in *L. alba* (Rocha et al., 2014) were screened for their transferability to 59 accessions of *L. sidoides* and *L. gracilis*. For all loci, the 5'-end of the forward primer was attached to an M13 tag (5'-CACGACGTTGTAAAACGAC-3'). The polymerase chain reac-
tions (PCRs) were performed in a 20-μL volume containing 2 μL genomic DNA (10 ng), 11.9 μL ultrapure water, 2.0 μL buffer Taq DNA polymerase [75 mM Tris-HCl, 20 mM (NH₄)₂SO₄, pH 8.8, 0.01% (v/v) Tween 20], 2.0 μL 25 mM MgCl₂, 0.2 μL 2.5U/μL Taq DNA polymerase, 1.0 μL 25 mM dNTPs, 0.35 μL 10 µM of each primer (forward + reverse), and 0.2 μL 10 µM labeled tag with the fluorophore IRDye700 or IRDye800 (LI-COR Biosciences, Lincoln, NE, USA). The PCR amplifications were performed with a touchdown program under the following conditions: an initial cycle at 94°C for 5 min; 10 cycles touchdown at 94°C for 40 s for denaturation, annealing temperature of each primer for 40 s (-1°C), and 72°C for 1 min for fragment extension; 30 cycles at 94°C for 40 s, 40°C for 40 s, and 72°C for 1 min; and a final extension cycle at 72°C for 10 min.

The products were initially verified on 2% agarose gels and then electrophoresed on an automated DNA sequencer LI-COR Model 4300 (LI-COR Biosciences) equipped with 2 infrared lasers with the ability to simultaneously read 2 wavelengths (700-800 nm), using the standard fragments (50-350 bp) labeled with fluorescence IRDye700 and IRDye800 (LI-COR Biosciences). The exact sizes of fragments were determined using the SAGA™ software version 3.3 (LI-COR Biosciences). The number of alleles, observed heterozygosity, and expected heterozygosity were estimated using the MStools software (Park, 2001). The deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were verified by Genepop (Rousset, 2008).

RESULTS AND DISCUSSION

Of 20 SSR loci, 12 (60%) were successfully transferred. Ten loci were moderately polymorphic in the AGB genotypes, and 11 loci were polymorphic in the collected *Lippia* accessions. A total of 23, 22, and 36 alleles, with an average of 2.30, 2.20, and 3.27 alleles per locus were identified for *L. sidoides* accessions in the AGB, *L. gracilis* accessions in the AGB, and *Lippia* spp accessions, respectively (Table 1). The expected heterozygosity ranged from 0.745 to 0.312, 0.626 to 0.143, and 0.755 to 0.141, while the observed heterozygosity ranged from 0.750 to 0.364, 0.857 to 0.143, and 0.917 to 0.103, for *L. sidoides*, *L. gracilis*, and *Lippia* spp accessions, respectively.

Six loci (LA08, LA09, LAD03, LAG05, LAB05, and LA02) departed significantly from HWE after Bonferroni’s correction (P < 0.05). However, accessions maintained in germplasm banks are not expected to be in HWE because they are collected from different populations and they do not mate (Bajay et al., 2009; Sandes et al., 2013). Loci in HWE are expected only in randomly mating populations (panmictic) after at least 1 generation of open pollination (Weir, 1996). The linkage disequilibrium was tested by adjusting the P value for the 5% nominal level, and no disequilibrium was detected among all loci.

The number of alleles and heterozygosity values confirm that the germplasm bank of *L. sidoides* and *L. gracilis* possesses considerable genetic variability, reinforcing the importance of conserving all genotypes. Some accessions of the collected *Lippia* spp were highly divergent, showing their importance and complementing the AGB. The described primers represent a useful tool for population genetics studies in *Lippia*. They can be used to verify the variability in other germplasm banks of *Lippia* spp, allowing the development of strategies for the conservation, management, and breeding programs of these important, endemic, medicinal, and aromatic species.
## Table 1. Sequences of the primer pairs providing amplification of microsatellite loci, GenBank accession numbers, repeat motif, annealing temperature (Ta), allele range, number of alleles (N), and expected (H_e) and observed (H_o) heterozygosities for *Lippia sidoides*, *L. gracilis*, and *Lippia* spp populations.

<table>
<thead>
<tr>
<th>Locus/GenBank No.</th>
<th>Primer sequence (5’-3’)</th>
<th>Repeat motif</th>
<th>Ta (°C)</th>
<th>P value</th>
<th>Lippia sidoides</th>
<th>Lippia gracilis</th>
<th>Lippia spp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Size range (bp)</td>
<td>N</td>
<td>H_e</td>
</tr>
<tr>
<td>LA01 JN626260</td>
<td>F: *CAGATTAGGGGTGGACAAA (CT)_8</td>
<td>53</td>
<td>0.0440</td>
<td>164-182</td>
<td>2</td>
<td>0.364</td>
<td>0.312</td>
</tr>
<tr>
<td>LA04 JN626263</td>
<td>F: *GCTTATGTTGGCATCTATGG (AC)_6</td>
<td>55</td>
<td>0.0070</td>
<td>208-216</td>
<td>2</td>
<td>0.583</td>
<td>0.431</td>
</tr>
<tr>
<td>LA08 JN626267</td>
<td>F: *TTCTGCTCATGCGCTTTGGATG (TA)_8(CGT)_10</td>
<td>55</td>
<td>0.0000</td>
<td>165-177</td>
<td>2</td>
<td>0.750</td>
<td>0.489</td>
</tr>
<tr>
<td>LA09 JQ806352</td>
<td>F: *GGGGCTACACAGACGATACA (AC)_5(T)CACG (TA)_9</td>
<td>55</td>
<td>0.0000</td>
<td>248-304</td>
<td>2</td>
<td>0.727</td>
<td>0.519</td>
</tr>
<tr>
<td>LAD03 KF611775</td>
<td>F: *CGACCTAAACACACACCTAAGCA (CT)_6 (AC)_8</td>
<td>55</td>
<td>0.0000</td>
<td>255</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>LAF04 KF611778</td>
<td>F: *GGCCTGTGGTGGTATGCTGTA (TA)_9(GT)_10</td>
<td>55</td>
<td>0.0148</td>
<td>171-179</td>
<td>5</td>
<td>0.000</td>
<td>0.745</td>
</tr>
<tr>
<td>LAB06 KF611778</td>
<td>F: *TACACTGCTTGGGATGGTTCG (ACG) (AG)_1</td>
<td>55</td>
<td>1.0000</td>
<td>91-103</td>
<td>3</td>
<td>0.417</td>
<td>0.649</td>
</tr>
<tr>
<td>LAF05 KF611781</td>
<td>F: *ACGTCTGGGATGGTCATGCGC (CA)_5</td>
<td>55</td>
<td>0.0000</td>
<td>253-257</td>
<td>2</td>
<td>0.500</td>
<td>0.391</td>
</tr>
<tr>
<td>LA05 KF611780</td>
<td>F: *TGCTGTTGTTGATGCTATACACCTA (ACG) (AG)_1</td>
<td>55</td>
<td>0.0000</td>
<td>115-135</td>
<td>3</td>
<td>0.583</td>
<td>0.518</td>
</tr>
<tr>
<td>LAB05 KF611774</td>
<td>F: *CACACTTGGGTTGATGATC (AC)_10</td>
<td>50</td>
<td>0.0012</td>
<td>115-135</td>
<td>3</td>
<td>0.583</td>
<td>0.518</td>
</tr>
<tr>
<td>LA02 KF611777</td>
<td>F: *GGTTGTCGTTGGTTGATGCGATG (TTAAT)</td>
<td>55</td>
<td>0.0000</td>
<td>225-235</td>
<td>1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>JN626261 R: GGGTTGACCAAAAAGTCACAA (GGTTAAT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAG04 KF611779</td>
<td>F: *GGTATCCATGGATGTTGCCTGAC (CA)_6</td>
<td>55</td>
<td>0.0000</td>
<td>206-232</td>
<td>2</td>
<td>0.417</td>
<td>0.344</td>
</tr>
</tbody>
</table>

* M13 tag (5’-CACGACGTTGTAAAACGAC-3’) label; F = forward primer; R = reverse primer, HWE = Hardy-Weinberg equilibrium; and *P value departs significantly from HWE at P < 0.05 after Bonferroni’s correction.
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